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A SEVEN-DAY LIFE-CYCLE CLADOCERAN TOXICITY TEST

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Abstract — A three-brood life cycle test for *Ceriodaphnia reticulata*, using renewal techniques, that can be completed in 7 d is described. The test is convenient when samples to be tested are limited in volume or when time is especially important, such as in on-site effluent testing. The cladoceran *C. reticulata* is easily cultured and is not bothered by handling; control survival in tests has not been a problem.

Keywords — Toxicity test Chronic daphnid test Cladoceran test Aquatic toxicity Life cycle test method *Ceriodaphnia reticulata* Culturing of cladocerans

INTRODUCTION

Cladocerans, especially *Daphnia magna*, are extensively used for aquatic toxicity testing because they are readily available, adaptable to laboratory conditions, require little space and are frequently one of the more sensitive aquatic animals to chemicals. The functional role of cladocerans in the community is less often mentioned as a reason for using them as test animals. They are among the most important groups converting phytoplankton and, perhaps more importantly, bacteria into animal protein that is nutritionally valuable for higher animals, such as fishes. Thus, their important role in aquatic communities makes them logical choices for inclusion among species that need to be protected.

Of the cladocerans, *D. magna* has been used in most aquatic toxicity testing for a variety of reasons. Undoubtedly, history and habit play a large role in their current importance, and the large data base already established is another factor. Their relatively large size, compared with other cladocerans, is a convenient feature.

There are some disadvantages of *D. magna* that must be considered. Perhaps most significant is that the species is virtually nonexistent in the fauna of North America. Opinion is divided about the proper diet for *D. magna*, and we have found it more troublesome to culture than the species discussed in this report. Test protocols such as the one developed by the U.S. Environmental Protection Agency (EPA) Office of Toxic Substances [1] use 20°C temperatures to offset mortality problems in the controls in chronic tests. The use of such temperatures requires longer tests to achieve sufficient reproduction than would

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be the case if 25°C were used. As a result of the Toxic Substances Control Act and other laws that directly or indirectly require toxicity tests, large numbers of tests are being performed. The problems with control survival and the need for longer tests as a result of cooler test temperatures are important cost factors for routine testing. Obviously, if tests were cheaper and shorter, more information could be required without unreasonable expense.

For tests that must be done under difficult working conditions or while staff are in transit, each additional day that tests must be extended adds much more cost than would be incurred in routine laboratory testing. When water samples must be shipped to remote laboratories for testing, longer tests usually require more water to be shipped, thus increasing costs.

The method described in this article was specifically designed for both on-site effluent tests, for which time in the field is costly, and for shipped-sample testing, for which volumes of water and waste must be minimized. The method also has been used successfully for single-compound testing. The small test volumes make it suitable for leachate testing, for which the available volume of test solution is small. The method employs the cladoceran *Ceriodaphnia reticulata*, which has a wide distribution and is abundant within its range [2]. It inhabits larger streams, reservoirs and lakes, and so is important in many types of habitats. During our 3 years of culture work, in side by side comparisons, *C. reticulata* has been easier to culture than *D. magna* and population stability has been much better. The first brood is produced from 72 to 120 h at 25°C and a three-brood life cycle test can be completed in 7 d. Tests can be extended if more broods are desired.

Since this work was completed, another species *Ceriodaphnia affinis/dubia* appeared in some cultures. We have found it to be an even better choice as a test animal because the young are easier to count, larger broods are produced and ephippia are even more uncommon in crowded cultures. All techniques are identical for both species.

In future publications, a flow-through test method will be described, as will the results of a 10-generation feeding study [3] to demonstrate the nutritional adequacy of the diet. The present article includes information on culture techniques, handling procedures, relative sensitivity and some special applications to effluent work, in addition to the test method itself. Each subject area has its own heading to aid the reader in finding various parts, and we have departed from the standard Materials–Results–Discussion format to make items easier to find when referring to the method.

CULTURE PROCEDURE

C. reticulata has been cultured at the Environmetal Research Laboratory–Duluth for nearly 4 years. During that period this species has been maintained in a flow-through system and fed bacteria, using trout food as a bacterial nutrient source. Efforts are currently under way to define numbers and species of bacteria being utilized by *C. reticulata* to improve knowledge of its nutritional requirements. For this renewal method and for culturing animals for it, a more concentrated food was needed.

The diet of *Ceriodaphnia* has been investigated by others. *C. quadrangula* did not reproduce when fed *Scenedesmus* alone, but did when there was an associated microbial population [4]. For *C. reticulata*, bacteria made up 18 to 26% of the diet in the Lower Don [5]. When *C. reticulata* were fed a mixture of algae and bacteria, there was preferential selection of bacteria over algae [6]. The literature is in general agreement that *C. reticulata* is primarily a bacterial feeder. A small-batch culture method to produce bacteria for feeding in the renewal tests was considered, but a more convenient food was preferable. Feeding tests were completed using a water suspension of activated dry yeast, the type commonly called baker's yeast. These preliminary tests were done

with Lake Superior water in which the animals quickly starve if not fed because algal and bacterial populations are so low. The animals appeared to do nearly as well on the yeast diet as in the flow-through cultures, although the number of young per female was somewhat less using the yeast diet rather than the flow-through test systems.

At this time, the exact food eaten by daphnids is not known. Undoubtedly, bacterial populations develop quickly using the yeast cells as a food source. The yeast does not remain suspended long but forms a visible film on the bottom. The daphnids are probably not able to feed on the film, but more work needs to be done to determine the exact food utilized. [Note: Recent experiences of other labs using reconstituted water, well water or dechlorinated tap water have found aging the water to be necessary. Aging very likely provides time for bacterial populations to increase to levels closer to these found in natural surface water. Three of these labs, two using well water and one using reconstituted water, feel the addition of Cerophyl® (Agri-Tech) increases nutritional adequacy.] Whatever the case, the yeast diet has the advantage of convenience, can be added in exact amounts in a repeatable manner and, based on young production, is adequate in most surface waters. Since yeast is convenient and readily available, as well as apparently suitable as a food source—although perhaps as an indirect one—it was chosen for this study. Experience may reveal an even better food.

Feeding tests were conducted for 10 generations to ascertain the nutritional adequacy of the yeast feeding regimen. Figure 1 contains data for three of these generations (F_0, F_3 and F_7) which show no decline in young production or longevity [3]. As soon as the feeding tests indicated that the yeast diet was a suitable food, static cultures were started to provide animals, accustomed to the same diet, that would be used for testing.

Cultures have been maintained on the yeast diet for 18 months. A culture dish 185 mm in diameter and 100 mm deep filled to a depth of 50 mm with approximately 1.5 liters of laboratory water (Lake Superior water) is used. Approximately 5 ml of a 5,000-mg/L yeast suspension is added daily. A culture is begun with 50 to 100 animals and maintained for 3 weeks. A new culture is started each week and three culture dishes are used so that each week one is discarded. By this simple rotation of three cultures, which requires only 10 min per week for maintenance, an abundant supply of adults is available for production of test animals. The population density very soon increases to the point at which food is limiting and young production falls. When young test animals are needed, adults are taken from the cultures and placed, one each, in 30-ml beakers with 15 ml of water and 250 μg of yeast (0.05 ml of 5,000-mg/L yeast suspension is added daily).[1] If animals are severely starved, the first brood will consist of two to six animals and several days will be needed before broods of 10 or more are obtained. This response time agrees with the literature, in which reports state that 38 to 40 h are required for egg development and release of young at 24°C [7]. Thus far, known-age adults have not been used for brood stock but young adults having small broods of three to six are not used.

Unlike *D. magna*, *C. reticulata* reacts to a food deficiency by reducing or stopping reproductive activity. The populations do not crash as do those of *D. magna* and the food-deprived adults do not seem to suffer ill effects. Food limitation can be avoided by cropping the population or using flow-through methods. Increased feeding in static cultures should be done with care because dissolved oxygen concentrations can be lowered by the additional food. Gentle aeration does not seem to adversely affect the animals and has been used to assure acceptable levels of dissolved oxygen.

[1]Some commercially available yeasts do not dissolve as well as others, and those that dissolve readily give better results. Fleischmann's yeast was used for this study.

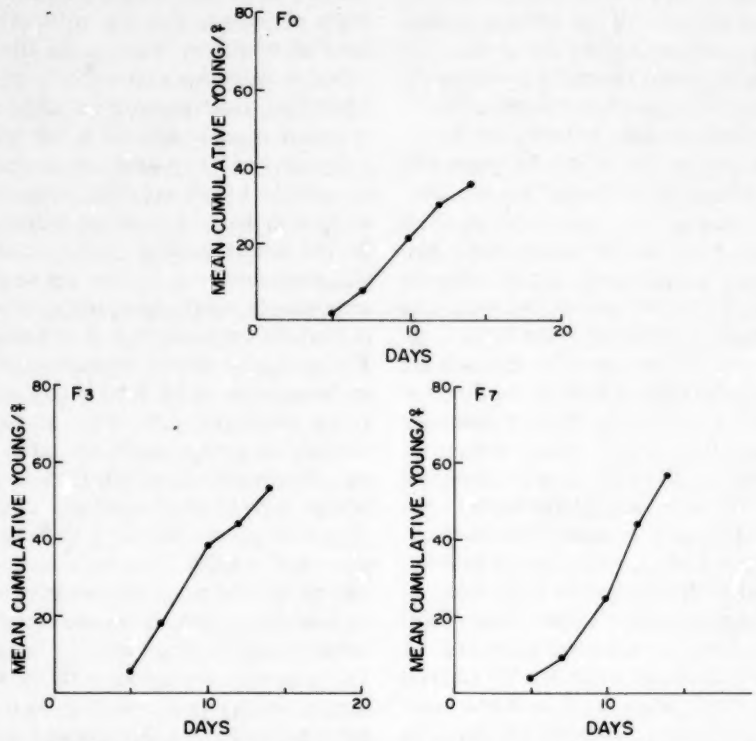

Fig. 1. Young production in the F_0, F_3 and F_7 generations fed 250 μg yeast per animal per day.

No special temperature control is provided, although the room temperature is stable and the culture temperatures stay at 24 to 25°C. Cultures have been kept at temperatures from 18 to 27°C and usual densities were maintained. Cultures maintained in light intensities bright enough to promote algal growth or in very dimly lighted conditions under which algae do not grow have done equally well. Periphyton growth in the lighted cultures helps increase oxygen concentrations and is an advantage.

At the present time, we are not suggesting criteria for defining animals acceptable for producing young for testing. Ephippia rarely occur in the cultures and are not induced in the food-limited cultures described above. Well-fed adults should have 10 or more young per brood after the first two broods, and should have a brood at least every 2 d. Our experience indicates that normal young production could be used as an index for acceptability of adults for producing test animals. Males have been found at various times in tests. In every case their presence followed some identifiable stress on the culture, such as temperature shock or poor water quality. Males become distinguishable by day 3 or 4. Their outlines are more elliptical than spherical, compared with the females, and they swim more rapidly at a more uneven speed and do not get as large as fully grown females.

HANDLING THE ANIMALS

C. reticulata is tolerant of handling, and no special procedures or care have been found necessary. The techniques commonly used for *D. magna* have been used here.

Pipettes of approximately 2-mm bore are acceptable for transferring adults and young alike. Animals can be handled rapidly, with only a reasonable amount of care taken to avoid bumping on the pipette end and on the test container walls. Instantaneous temperature changes of 2°C seem to have no effect, and to date no problems with surface tension causing animals to float have been evidenced, although other species in the same conditions have exhibited severe problems at times, especially *D. pulex*. Instantaneous pH changes of more than 0.5 units have killed some animals in tests in which pH was being altered, so something less, perhaps 0.2 units of change, should not be exceeded.

Some workers prefer to use a microscope for handling. The adult is slightly larger than a newborn *D. magna*. Neither the adults nor the young swim as rapidly as *D. pulex* and are therefore easier to catch. The young are as easy to see as young *D. pulex*. If the water containing the young is held so that light is transmitted vertically upward through it and the animals viewed against a dark background, the young appear very white and are easily seen without magnification. Each one can be examined in the pipette without magnification using the same lighting techniques.

During tests, however, viewing the young through a microscope is a good practice so the development of young and ad-normalities can be observed. Especially in certain natural waters and effluents, growth of fungi may physically entrap the animals, causing death that is not due to toxicity. *C. reticulata* has been found to be more tolerant of such problems than *D. magna* and *D. pulex*.

THE RENEWAL TEST METHOD

The testing is done in 30-ml beakers with 15 ml of test solution. The geometry of this combination allows one to view the entire volume of test water under a dissecting microscope without refocusing and with only slight horizontal movement of the beaker. Furthermore, the surface-to-volume ratio is such that dissolved oxygen has remained above 5 mg/L in all the tests completed to date, except when the BOD of several wastes was exceptionally high.

Ten beakers with one animal each are used for each test concentration and control. Tests should be started with young animals that are as similar in age as is practical. We have used animals that are not more than 2 h different in age. Usually 0- to 2-h-old young are used, but sometimes a group of animals within a 2-h age span difference, for example 6- to 8-h old, have been used when testing is delayed. This does not increase the variability of young production within the test. Producing enough young in such an age span has not been difficult. If the brood animals are put singly in 30-ml beakers, one can easily keep track of the age of all young and the appropriate beakers can be composited to make the lot of test animals for distribution in the test. If animals are well fed, 15 or 20 adults carrying eyed young are enough to produce 60 young within a 2-h age span. Experience has shown that 150 adults, each in a 30-ml beaker and fed daily, are enough to produce 60 young every 2 h.

The entire test has been designed to fit into the normal 5-d work week to reduce the cost of the test due to weekend work. Accordingly, if the test is started on Monday (day 1) the adult is moved into new test solutions and the young counted and discarded at the same time on Wednesday (beginning of day 3) and Friday (day 5). The test is terminated on the following Monday (end of day 7) by counting the number of young. Obviously the day the test is begun and changed is immaterial, but this schedule fits well into a normal work week. There is some advantage if the test is started on Friday and the first change made on Monday, because the longer period between changes occurs when the test animals are the smallest and no young are present. So long as the changes are made on Monday, Wednesday and Friday, the only necessary weekend work is

feeding, which requires only a few minutes each day.

If difficulty is encountered in counting young after the adult is removed, a drop of concentrated acid (such as H_2SO_4) can be added. The beaker should be checked for mortality of young before the acid is added. The acid will quickly kill the young and they will settle to the bottom, where counting them is easy. Various broods can be identified by size if desired. At the end of day 7, adult survival and a count of young are needed to end the test. An average of 2.5 broods per surviving adult in the controls has been used as the endpoint of the test. Acceptable control mortality of the adults can be set at 20% in accordance with EPA [1] methods, or, a more rigorous value of 10% is not difficult to achieve.

The animals need to be fed every day, and especially on days 5, 6 and 7, when both young and adults are present and consuming foods. A 5-mg/ml solution of dry yeast dissolved in water is fed at the rate of 0.05 ml per day per beaker. Drop sizes from medicine droppers vary, but droppers can be found that will deliver drops of approximately 0.05 ml. This gives 250 μg of yeast per animal per day and results in an initial concentration of 16.7 mg/L of suspended solids, which is within the suspended solids guidelines of such methods as the EPA protocol [1]. In testing preliminary to the feeding study referred to above, there was evidence that more yeast resulted in larger brood sizes. The 250-μg level was selected so that suspended solids limits of other methods [1,8] were observed and to minimize dissolved oxygen depression and toxicant interactions. If test water contains sizable populations of bacteria, as many surface waters do, then more young will be produced when 250 μg yeast per animal per day is given than when a relatively sterile water, such as well water, is used.

The first brood is released between 72 and 120 h, and usually contains three or four young. Depending on feeding level and temperature, the second and third broods will follow at 24- to 36-h intervals. In waters such as Lake Superior water, in which bacterial counts are relatively low, 15 young in three broods is average, and we have arbitrarily used nine as the minimum mean number that is acceptable [3]. However, young production will increase to 25 to 30 per female if high bacterial populations are present in addition to the yeast. Domestic sewage treatment plant effluents that are not toxic will likely produce young in such numbers. When testing effluents mixed with dilution water of a greatly different trophic condition, dilution of the available food may affect the expression of toxicity.

To date, we have used analyses of variance (ANOVA) and multiple range tests to establish no-effect and effect exposures for young production. Adults (the original test animals) that die are still counted in obtaining the estimate of young per female because the adult mortality when the brood is shed is often the most sensitive indicator of effect. To exclude this mortality in calculating young production would cause effects to be missed. However, the inclusion of the mortality causes standard deviations to be large and probably not normally distributed. If so, then ANOVA is not appropriate without transforming the data. A better statistical test is needed.

Table 1 gives control young production and survival for several tests in surface waters having various bacterial populations. The standard deviations are large for some tests. No attempt has been made as yet to reduce these by the use of such techniques as selecting brood stock for uniform young production and using known-age adults to produce young for tests. Until methods to obtain lower standard deviations are found, an increase of test animals per treatment may be necessary whenever better resolution of treatment differences is needed. The reader can be guided by the variability shown in Table 1 to determine if a larger N is necessary for any specific test. Table 2 contains the results of an effluent test using

Table 1. Reproductive data for control animals taken from various effluent tests

Water source	Final survival (%)	Mean no. (SD) of young per female	Mean no. broods per female
Lake Superior	90[a]	12.5 (6.3)[b]	2.5
Scippo Creek	90	18.5 (6.6)	2.7
Ottawa River	90	15.5 (8.0)	2.1
Lake Superior	80	15.9 (9.8)	—[c]
Raisin River	90	23.6 (10.4)	—[c]
Skeleton Creek	90	23.4 (9.8)	—[c]
Boggy Creek	100	14.7 (2.8)	—[c]
Ottawa River	100	26.6 (5.5)	2.8
Ottawa River	100	25.8 (4.6)	2.9

[a]n = 10.
[b]Number of young is based on 10 animals, regardless of amount or time of mortality.
[c]Broods not determined for these specific treatments.

receiving water as the diluent. Where effluent testing was done and there was reason to expect variability of effluent quality, the test solutions were changed every day to expose test organisms to a more representative sample. No increase in adult mortality or decrease in young production was noted. Those tests using Scippo Creek, Ottawa River and Lake Superior water were changed daily, and the standard deviations and mean number of young appear similar to those in tests for which only two changes were made.

For five concentrations and a control, with 10 animals per concentration, about 1 h is required for making new solutions, counting young and moving the adults. For the regular test with two changes of test solution, 3 to 4 h of work are involved in setting up the test, renewing the solutions and terminating. This time does not include chemical analyses for the toxicant. About 50 chronic tests have been completed on effluents, and this time estimate has been typical, even for on-site situations. Further savings of time has been achieved recently by use of innovations for preparing and dispensing test solutions. Figure 2 shows a simple device that can dispense 15 ml of test water into each of ten beakers simultaneously. This device reduced the time required to change a test with five concentrations plus a control by 10%. It consists of a small trough with test tubes extending through the bottom. Each tube is calibrated to the 15-ml mark and a cut is made at the mark. The tube is then positioned so that the lower edge of the opening of the tube is aligned with the bottom of the trough. About 160 ml of test water is poured into the trough and the trough is tilted from end to end until all tubes are filled. Then the tubes are emptied into a row of 10 beakers positioned by a holder and the excess 10 ml is spilled from the trough into a shallow tray for later disposal.

The small test volumes do pose some problems for chemical measurement. Dissolved oxygen probes and pH electrodes are usually small enough to fit into the beaker. Hardness, alkalinity and analytical measures of test substances must be done on a

Table 2. Seven-day renewal test with an industrial effluent and *C. reticulata*

Effluent Concentration (%)	Mean no. (SD) of young per original female	No. of broods per female	7-d survival of original females (%)
100	0.0	0.0	0
30	3.9 (3.6)	3.1	100
10	25.6 (6.1)	3.5	100
3	26.4 (6.1)	3.3	100
1	21.9 (6.0)	3.0	100
Control	22.5 (3.6)	3.2	100

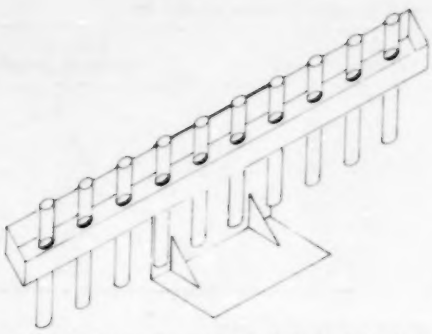

Fig. 2. Device for dispensing 15 ml of test solution into beakers for the life cycle test.

composite sample from the combined volume of the 10 beakers. Usually, hardness and alkalinity are best measured on additional test solutions made for that purpose, leaving the 150 ml for toxicant analysis after the animals are removed.

RELATIVE SENSITIVITY

Table 3 contains comparative 48-h LC_{50} data for four cladoceran species. The tests are all static–nonmeasured except as noted. The dilution water was Lake Superior water, with a hardness of 45 mg/L and alkalinity of 43 to 45 mg/L, both as $CaCO_3$. The pH was 7.2 to 7.4 and the water contained 50,000 to 100,000 bacterial cells per milliliter. These bacteria were available as a food source, making these tests different from acute tests done in sterile water without feeding. Two replicates of five animals each, at each concentration, were tested. Control mortality was 20% or less in every case, except for the lead test and *D. magna*. *D. magna* and *D. pulex* were tested in 100 ml of water and *Simocephalus vetulus* and *Ceriodaphnia* in 15 ml. The *Ceriodaphnia* were less than 4 h old and the others less than 24 h old at the start of the test.

One can see that *C. reticulata* was notably more sensitive to lead than *D. magna*, and that *D. pulex* was more tolerant to hexachloroethane than the other three species. Generally though, the species did not differ in response by more than a factor of 2. These data suggest that none of the species are distinctly more sensitive.

AMBIENT TOXICITY MEASUREMENT

Heretofore, the measurement of the chronic toxicity of water samples taken from receiving waters below discharge points has been very expensive because of the time and volume of water required. We have used this 7-d chronic test in mobile laboratories on-site to measure toxicity in receiving water after the effluent has mixed into the water column. By sampling at intervals downstream, the die-away of toxicity can also be measured to gain an estimate of its persistence. (Note: The authors are not implying that the cause of toxicity die-away can be delineated. Degradation, sorption on sediment, complexing, dilution and many other processes may cause toxicity to disappear.) Using this approach, six stations can be evaluated, with a work load equivalent to one standard test with five concentrations and a control. We have had difficulty with fungal growths entrapping the test animals at only one site. Table 4 shows the results of an ambient toxicity test over 22 miles of a stream with three effluent discharges into it.

By being able to measure chronic toxicity on-site, in 7 d for seven stations in an 8-h working day, we can now assess receiving water toxicity and do so without having to use the application factors and other extrapolations that must be used when only acute data are obtainable. Alternatively, application factors for specific wastes in receiving water can be measured cheaply for use with resident-species LC_{50} values to estimate no-effect concentrations for indigenous fauna.

SUMMARY

The test species and protocol described above offer an opportunity to reduce by one-half to two-thirds the length of time required to perform a cladoceran life cycle test, as compared to tests using *D. magna*. In addition, *C. reticulata* is commonly found in the United States, whereas *D. magna* is not.

Table 3. Comparative sensitivity for four species of cladocerans to 13 substances [a]

Toxicant	*D. magna*	*D. pulex*	*C. reticulata*	*S. vetulus*
Ammonia (as NH_3)	189 (131–273)	187 (151–231)	131 (115–151)	123 (92–166)
Pentachlorophenol	0.143 (—)	0.246 (—)	0.164 (0.129–0.209)	0.217 (0.171–0.276)
Hexachloroethane	2.7 (2.0–3.6)	>10.0	3.3 (2.3–4.7)	5.8 (3.7–9.1)
Tetrachlorophenol	0.406 (.319–.516)	1.000 (—)	0.334 (0.286–0.390)	0.145 (0.095–0.222)
2-Chloroethanol[b]	308 (258–367)	574 (470–702)	250 (191–326)	—
2,4-Pentanedione[b]	35.4 (—)	>50	>50	>50
Arsenic	3.8 (3.3–4.3)	1.9 (1.7–2.2)	1.8 (1.5–2.2)	1.7 (—)
Cadmium	0.118 (0.089–0.155)	0.068 (.053–.088)	0.066 (0.056–0.078)	0.024 (0.019–0.030)
Lead	4.4 (3.6–5.3)	5.1 (—)	0.53 (—)	4.5 (3.6–5.5)
Silver	—	0.014 (—)	0.011 (0.008–0.014)	0.015 (0.013–0.018)
Zinc	0.068 (—)	0.107 (0.076–0.151)	0.076 (0.061–0.093)	—
Copper	0.054 (—)	0.053 (0.046–0.061)	0.017 (0.014–0.021)	0.057 (0.052–0.064)
Hexavalent chromium	.022[c] (—)	0.048 (0.037–0.064)	0.045[c] (—)	0.050 (0.039–0.065)

[a]48-h LC_{50} concentrations in mg/L except as noted; 95% confidence limits in parentheses.
[b]Expressed in μl/L.
[c]Values from flow-through test with measured concentrations.

Table 4. Ambient toxicity in the Ottawa River, Ohio

Stream station	River mile	Mean no. (SD) of young per female
2 (Discharge No. 1)	37.7	29.2 (7.1)
3 (Discharge No. 2)	37.4	2.7 (2.5)
4 (Discharge No. 3)	36.9	11.9 (4.6)
5	35.4	8.4 (5.3)
6	32.6	4.2 (2.6)
7	28.8	6.8 (5.6)
8	16.0	27.9 (3.3)

The test is especially useful when expensive on-site effluent work must be done or when the volume of sample and /or dilution water available is small.

In the experience of the authors, the species is much easier to culture and fewer mortality problems have been encountered than for *D. magna*. Space requirements are very minor and the test can be performed with reasonable equipment cost.

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